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AN IMPROVED BRILLIANT-GREEN CULTURE MEDIUM FOR THE ISOLATION OF TYPHOID BACILLI FROM STOOLS *

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The great variety of methods that have been recommended for the isolation of typhoid bacilli from stools is strong evidence that none of these methods is thoroughly satisfactory.

Three general types of media have been devised for this purpose:

1. Solid media on which there is a sharp differentiation between lactose-fermenting and non-lactose-fermenting colonies, but no inhibition of the growth of organisms accompanying the typhoid bacilli. The Conradi-Drigalski and Endo plates have displaced all others in this group. Harris and Teague¹ have recently devised a methylene-blue eosin plate which also belongs in this group and which has certain advantages over the two plates just mentioned.

2. Solid media which inhibit the growth of many strains of *B. coli* and of other fecal bacteria to a much greater extent than they inhibit *B. typhosus*. Many varieties of malachite-green agar and Conradi's brilliant-green agar belong here. These media were popular for a while but gradually fell into disuse for reasons which will be discussed later in this paper.

3. Fluid enriching media which allow the typhoid bacilli to multiply more rapidly than the accompanying fecal bacteria. Caffein broth, bile, and Brown-ing's brilliant-green peptone solution represent attempts in this direction, but they must be regarded as failures.

Brilliant green was found by Conradi to have a marked inhibitive action on *B. coli* at dilutions which would allow typhoid bacilli to grow, and its action in this respect was more selective than that of any other dye tried by him. Krumwiede and Pratt state that a number of the green dyes show this differential action, but that at the appropriate dilution no one of them is more differential than another. In our hands solid green dissolved in nutrient agar was as selective as brilliant green, but the malachite greens were inferior. It may be accepted, therefore, that as yet no stain has been found to have a greater selective action toward the typhoid bacillus than has brilliant green.

But while the majority of strains of *B. coli* are more readily inhibited by brilliant green than is the typhoid bacillus, yet the reverse is not infrequently the case and for the aerogenes group this seems to be the rule rather than the exception.

Altho brilliant green is far more selective in this sense than are the related violet dyes, nevertheless certain strains of *B. coli* that are resistant to brilliant green are inhibited by some of the latter stains. Furthermore, these violet dyes differ among themselves, some inhibiting one strain of *B. coli*, some

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¹ Jour. Infect. Dis., 1916, 18, p. 596.

another, as is illustrated in Table 1. Pure cultures of strains of *B. coli* known to be resistant to brilliant green were suspended in salt solution, one loop of which was inoculated on each of the plates. The largest amount of stain which, dissolved in nutrient agar of reaction + 1, allowed *B. typhosus* to grow, was employed in each instance. The readings were made after 48 hours' incubation; zero thus indicates complete inhibition of growth.

It is seen from the table that the several strains behave differently in the presence of different dyes. These are of course selected strains; many strains that are resistant to brilliant green are resistant to all of these violet dyes as well.

It seemed likely, therefore, that by adding certain of these stains in proper amounts, to brilliant-green agar, there would be obtained a much more complete inhibition of the fecal bacteria. Many such mixtures have been prepared by us, but none of them has yielded as good inhibition when inoculated with feces as can be secured with brilliant-green agar alone.

Brilliant green dissolved in ordinary nutrient agar of reaction + 1 to the amount of 1/3000% makes an excellent medium for the isolation of typhoid bacilli from the majority of stools. It is superior to Conradi's brilliant-green medium, since the increased acidity of the latter and the picric acid contained diminish rather than heighten the selective action of the brilliant green. The weakness of brilliant-green agar lies in the fact that when a specimen of feces containing colon bacilli resistant to brilliant green is encountered, numerous colonies develop on the plate, and, as there is but little difference between the appearance of some of these colonies of *B. coli* and that of colonies of *B. typhosus*, the result is less satisfactory than on the Endo plate. Another point of importance is that the margin of safety with regard to the amount of brilliant green to be used is not wide: if too much is employed the typhoid colonies develop slowly—sometimes only after 48 hours' incubation—and are reduced in number; if too little, the colon bacilli are not adequately inhibited. Furthermore, different lots of agar containing brilliant green in exactly the same concentrations may yield somewhat different results. A cloudiness in the agar renders a certain amount of the brilliant green inactive and so it is conceivable that small particles distributed throughout the agar in quantities insufficient to produce a noticeable cloudiness might yet absorb an appreciable amount of the stain. In spite of these dangers brilliant-green agar yields excellent results with most stools, allowing the inoculation of comparatively large amounts of the feces and showing often only typhoid colonies or typhoid colonies and a few opaque colonies readily distinguishable from them. But since one cannot know beforehand whether or not a given stool is going to be the exception and yield too many colonies of *B. coli* on the brilliant-green plate, one hesi-

tates to use this plate as a routine procedure. We have had no experience with the various malachite-green media that have been recommended, but we judge that they are inferior to the brilliant-green agar and that the same dangers attend their use. This would explain their gradual lapse from popularity to disfavor as their shortcomings were appreciated. There is no doubt that brilliant-green agar employed together with the Endo plate will yield much better results than the Endo plate alone.

TABLE 1
THE VARIATION AMONG DYES IN THEIR INHIBITION OF DIFFERENT STRAINS OF *B. COLI* AND *B. TYPHOSUS*

Culture	Brilliant Green .0005%	Victoria 4 R .025%	Ethylviolet .025%	Methylviolet 6 B .025%	Dahlia .025%
<i>B. typhosus</i>	+	+	0	+	+
<i>F. endo</i> 2....	+	+	0	+	+
Aerog. <i>J. B.</i>	+	+	+	0	0
<i>T. d.</i>	+	+	0	+	+
Aerog. 4.....	+	0	+	+	+

We have devised a medium which has practically the same selective action for the typhoid bacillus as brilliant-green agar and which at the same time differentiates between the lactose-fermenting and the non-lactose-fermenting colonies. The following experiment illustrates the principles on which the method rests:

Ordinary nutrient agar containing meat infusion, 1% peptone, 0.5% sodium chlorid, and 1.5% agar was prepared in the autoclave, cleared with egg, and put into flasks. It was then sterilized in the autoclave and stored for use. After sterilization it titrated +0.9. The agar was melted and 1% of saccharose and 1% of lactose were added to it. A 1% solution of brilliant green in 50% alcohol and a 3% solution of yellow eosin in distilled water were prepared. Further dilutions of brilliant green in distilled water were made so that 1 c.c. of each added to 50 c.c. of agar yielded the final dilution of brilliant green indicated in the table. The eosin solution was added to some of the agar in the proportion of 1 c.c. to 50 c.c. and the brilliant-green solutions were added to this eosin agar to yield the final concentrations of brilliant green indicated in the table; namely, 0.003%, 0.005%, and 0.01%. Plates were poured from each dilution of the stain in agar as soon as it was prepared.

To another portion of this same lot of nutrient agar a thick suspension of feces, previously sterilized in the autoclave, was added until the whole mass of agar looked somewhat cloudy. Dilutions of bril-

liant green, and of eosin and brilliant green, were prepared in this agar plus feces in exactly the same manner as in clear agar, and plates were poured as before. Before inoculation the excess of moisture was removed by placing the uncovered plates face down in the incubator for about 20 minutes.

Portions of several fresh normal stools, rubbed up in salt solution, were filtered through a thin layer of absorbent cotton, and the mixture was then diluted 1:10 and 1:100. A loop of each of these suspensions was inoculated on a quadrant of each of the plates and a loop of a freshly prepared suspension of typhoid bacilli on the last quadrant. The number of colonies in each quadrant was recorded after 24 hours' incubation and again after 48 hours' incubation. Only the latter reading is given in the table.

TABLE 2
RESULTS OF A TEST OF BRILLIANT-GREEN CULTURE MEDIA FOR THE ISOLATION OF
TYPHOID BACILLI

Media		Mixture of Normal Stools			Suspension of Typhoid Bacilli
		1:100	1:10	1:1	
Brilliant-green with sterilized feces	.0003%	42	Numerous	∞	78
	.0005%	27	Numerous	∞	65
	.001 %	0	3	10	60
	.002 %	0	0	9	0
	.004 %	0	1	0	0
Brilliant-green control	.0003%	0	1	4	57
	.0005%	0	1	0	0
	.001 %	0	0	0	0
Eosin + brilliant-green with sterilized feces	.003 %	1	3	50	70
	.005 %	0	0	8	76
	.01 %	0	1	2	69
Eosin + brilliant-green control	.003 %	0	0	6	83
	.005 %	0	0	1	67
	.01 %	0	0	0	40
Plain agar control.....		34	160	∞	85

It is seen from Table 2, first, that the toxicity of brilliant green for the typhoid bacillus is reduced about tenfold by the addition of the eosin; second, that the inhibition of the colon bacilli of the stools is impaired to a much greater degree in the brilliant-green plates than in the eosin brilliant-green plates by the addition of sterilized feces to the medium. Thus, there is practically complete inhibition at 0.0003% brilliant green in the clear agar, while 3 times as much brilliant green (0.001%) is required to produce this result in the agar clouded with sterilized feces. With eosin a given amount of brilliant green produces only slightly less complete inhibition in the clouded

agar than in the clear. A third important fact, which is not brought out in the table, is that the typhoid colonies are quite different in color from the colonies of *B. coli* on the eosin brilliant-green agar.

The eosin brilliant-green agar containing lactose and saccharose is the medium which we herewith recommend for the isolation of typhoid bacilli from stools. In the article following this² we give a number of tables illustrating the mode of action of this plate, from which it is seen that dilutions of the various typhoid stools used which yield numbers of colonies of *B. coli* on the Endo plate and on the eosin methylene-blue plate of Holt-Harris and Teague³ usually show only a few colonies besides the typhoid ones on the eosin brilliant-green plate. Time and again typhoid bacilli were recovered from the eosin brilliant-green plate with no difficulty at all when the other two plates, inoculated with the same material, gave negative results. The eosin brilliant-green agar allows the typhoid colonies to develop practically as rapidly as they do on plain nutrient agar, and at the same time shows good inhibition of *B. coli*. After 18 hours' incubation the typhoid colonies by reflected light are grayish in color, while the colonies of *B. coli* have red centers. By transmitted light the typhoid colonies are transparent and colorless, while colonies of *B. coli* show dark centers. On further incubation the typhoid colony becomes pink and the whole colony of *B. coli* becomes dark-red or dark-purple in color; the typhoid colony remains translucent, while the colony of *B. coli* becomes more and more opaque. The typhoid colony may show the center somewhat darker than the periphery, but the whole colony is much paler than that of *B. coli* and is readily distinguished from it. If the colonies are packed closely together on the plate, this differentiation is obscured, the typhoid colonies assuming a darker color and those of *B. coli* failing to develop centers properly; however, on account of the inhibition which the medium exerts on the growth of most strains of *B. coli*, this is not likely to occur.

We believe that this medium is more satisfactory for the isolation of typhoid bacilli from stools than any other hitherto recommended.

We prepare the medium as follows: 500 gm. of chopped beef are placed in 1 liter of distilled water and kept in the ice-box over night. The infusion is squeezed through cheese cloth, heated in the Arnold sterilizer, and passed through filter paper. Witte's peptone (1%), chemically pure sodium chlorid (0.5%), and agar (1.5%) are added to the warm infusion, the peptone being first rubbed up into a paste in a little warm water. The flask of medium is

² Jour. Infect. Dis., 1916, 18, p. 653.

³ Ibid., p. 596.

then heated in the autoclave for 30 minutes at 120 C. The reaction is adjusted to +1 by the addition of 2 normal sodium hydrate and then the medium is heated a half hour in the Arnold sterilizer. The medium is again titrated and the reaction is brought to +1. It is cooled to 55 C., cleared with egg white, and filtered through cotton. It is then placed in flasks in amounts of 100 c.c. or 200 c.c., and heated for 20 minutes in the autoclave at 120 C. It is then stored, ready for use.

In pouring the plates a flask of this agar is melted and its reaction controlled. Lactose (1%) and saccharose (1%) are added. To every 50 c.c. of the agar is added 1 c.c. of a stock 3% solution of yellowish eosin. From the stock 1% brilliant-green solution in 50% alcohol, a $\frac{1}{6}$ % solution in distilled water is prepared and 1 c.c. of this is added to every 50 c.c. of the agar containing the eosin. After the stains have been distributed uniformly throughout the agar by shaking, plates are poured. These may be inoculated immediately after the agar solidifies, or they may be kept in the ice-box several days and then used.

Until one has become familiar with the appearance of the typhoid and colon-bacillus colonies on this plate and with the general behavior of the medium, control Endo plates or eosin methylene-blue plates should be used. We have recommended 3/50% eosin and 1/300% brilliant green. Agar as prepared in different laboratories possesses somewhat different characteristics and may require slightly more or slightly less brilliant green to yield optimal results. If 1/300% brilliant green does not give good inhibition of *B. coli* with agar prepared in a certain way, 1/275% brilliant green will probably do so without retarding the growth of *B. typhosus*. Hence, preliminary tests should be made with typhoid stools, or, if these are not available, with a freshly isolated strain of *B. typhosus* which has not been in contact with any stains, to determine the optimal amount of brilliant green, the eosin being kept constant in amount; thereafter the agar should always be prepared in the same way from similar materials and this amount of brilliant green used.

As we have not obtained good results on substituting Liebig's meat extract for the meat infusion, we warn against the use of the extract. By employing a meat infusion rendered sugar-free by incubation with *B. coli*, we have obtained beautiful differentiation of the colonies and excellent inhibition of *B. coli*, but the typhoid colonies were very small after 24 hours' incubation, and were somewhat reduced in number. After 48 hours' incubation this plate gives good results. On account of the greater trouble in preparing this medium and of the delay in obtaining results, the nutrient agar first described is to be preferred.

A comparative study of this medium, the Endo medium, and the eosin methylene-blue medium is presented in the succeeding article.